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ORIGINAL ARTICLE

Anti-obesity effects of kaempferol by inhibiting adipogenesis and increasing lipolysis in 3T3-L1 cells

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Abstract

Kaempferol is a natural flavonoid widely found in fruits, vegetables, and tea. Kaempferol possesses beneficial biological properties such as anti-inflammatory and antioxidant activities. Positive energy balance during obesity correlates with a pro-inflammatory chronic state. In this context, we hypothesized that kaempferol might promote anti-obesity effects by modulating adipogenesis and lipolytic pathways. Adipocyte viability at 24, 48, and 72 h was measured by an ATP-based assay. Pre-adipocytes (day 0) or mature adipocytes (day 12) were treated with 60 μM kaempferol until day 21 to evaluate its potential anti-adipogenic and lipolytic effect, respectively. Total lipid accumulation was assessed using Oil Red O staining assay. Gene expression was measured by RT-qPCR to evaluate the effect of kaempferol on adipogenesis and lipolysis gene expression. Our results showed a dose-dependent effect of kaempferol treatment on cell viability promoting cell death at higher than 60 μM concentration. Pre-adipocytes stimulation by 60 μM kaempferol resulted in 62% adipogenesis inhibition whereas in mature adipocytes, it reduced 39% intracellular lipid accumulation. Also, 60 μM kaempferol treatment decreased *Cebpa* mRNA expression when compared to control cells. In contrast, *Pnpla2* and *Lipe* gene expression were upregulated in 3T3-L1 cells incubated with 60 μM kaempferol. In summary, our results showed that kaempferol modulates adipogenic differentiation in 3T3-L1 cells by promoting downregulation of *Cebpa* gene expression and decreasing lipid accumulation in mature adipocytes by its positive effects on *Pnpla2* and *Lipe* mRNA levels. Kaempferol might display an anti-obesity effect.

Keywords Flavonoids · Kaempferol · Adipogenesis · Lipolysis · Obesity

Introduction

According to the World Health Organization (WHO), obesity is considered an abnormal or excessive fat accumulation in the adipose tissue [1]. Nowadays, obesity is one of the most serious challenges of public health being closely related to the development of an array of metabolic diseases such as diabetes mellitus, hypertension, atherosclerosis, and dyslipidemias [2].

Positive energy balance leads to an increase in adipose tissue accumulation during obesity which is influenced by many factors including an excessive caloric intake, energy expenditure, metabolic and endocrine molecules, genetic susceptibility, and environment [3]. Adipose tissue accumulation involves two metabolic and cellular processes: an increase in the quantity of lipids inside adipocytes (hypertrophy) and new adipose cells (hyperplasia) also known as adipogenesis [4–6]. Adipogenesis is modulated by signaling cascades involving bone morphogenetic protein-4 and peroxisome proliferator-activated receptor (PPAR) β/δ, which modulates the gene expression of PPARγ and the CCAAT-enhancer binding protein (C/EBP) family [7–10]. PPARγ and C/EBP are key transcriptional regulators of lipid homeostasis, which modulate the gene expression profile associated with fat accumulation, fatty

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acid transport, and lipolysis including fatty acid synthase (FASN), adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL) [11, 12].

Natural compounds can exert anti-obesity effect because of their potential to modulate metabolism [4]. Flavonoids are a group of natural substances that share a molecular structure characterized by having one or more phenolic rings. Flavonoids are widely distributed in plants, fruits, vegetables, tea, and wine [13]. In vitro and in vivo studies have identified anti-obesity effects including reduction of intestinal lipid absorption, inhibition of digestive enzymes, increased energy expenditure, decreased pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation [3, 8, 15, 16].

Kaempferol is a flavonoid found in several natural sources including apples, onions, broccoli, tomatoes, citrics, grapes, and berries [17]. Experimental evidence has substantially supported the benefitial effects of kaempferol including antioxidant, anti-inflammatory, antidiabetic, anticarcinogenic, and anti-obesity effects [18–23]. However, it is still unknown the selective molecular mechanism regarding the potential effect of kaempferol as an anti-obesity candidate.

In this study, we used 3T3-L1 cells to investigate the physiological and molecular mechanisms involved in the anti-obesity effect of kaempferol.

Materials and methods

Chemicals

Kaempferol was obtained directly from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) to make 10 mM stocks solutions and stored at 4 °C until their use.

Cell culture and treatments

3T3-L1 pre-adipocyte cell line was purchased from the American Type Culture Collection (ATCC) and expanded in Corning® T75 cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM, high glucose 4.5 g/l; supplemented with 10% (vol/vol) newborn calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and grown at 37° under a humidified 5% CO_2 atmosphere. Adipocyte differentiation was induced by treating 2 days post confluent cells (designated as day 0) with medium DMEM supplemented with 10% of fetal bovine serum (FBS) and 1 μL/ml of insulin, isobutyl-methylxanthine (IBMX), and dexamethasone (differentiation medium). After 2 days, the medium was replaced for fresh medium containing DMEM supplemented with 10% FBS and 1 μL/ml of insulin (post-differentiation medium), this medium was switched every 2 days until the end of the experiment (day 21).

Pre-adipocytes were treated from day 0 with 60 μM of kaempferol to assess the inhibitory potential for adipogenesis. On the other hand, from day 12, kaempferol was added to mature adipocytes to evaluate their lipolytic potential.

Cell viability assay

Cell viability was performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). 3T3-L1 cells were seeded into 96-well plates at a density of 10,000 cells/well and treated with dose-dependent kaempferol concentrations (20, 40, 60, 80, 100, 120, 150, 170 μM) for 24, 48, and 72 h. Kaempferol cytotoxicity was measured in the luminometer (Turner BioSystems Veritas Microplate Luminometer) following incubation.

Oil Red O staining

After treatments, cells were stained with Oil Red O solution using the Adipogenesis Assay Kit Cell-Based (Abcam) following the manufacturer's instructions. The accumulation of cellular lipid droplets was conducted by comparing kaempferol-treated cells with vehicle control-treated cells. In brief, cells were washed twice with Lipid Droplets Assay Wash Solution, subsequently, Lipid Droplets Assay Oil Red O Solution was added to the cells, and were incubated at room temperature for 20 min. After incubation, cells were visualized using a phase-contrast microscope and the stained lipid droplets were measured by reading absorbance in the spectrophotometer (BIO-RAD, iMark, Microplate Reader) at 490 nm.

RNA extraction and RT-qPCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentrations and quality were measured by NanoDrop 8000 (Thermo Scientific) whereas RNA integrity was determined by electrophoresis on 1% agarose gels stained with ethidium bromide, and visualized under UV light. RNA samples (9 ng/uL) were reverse-transcribed to cDNA using GoScript™ Reverse Transcription System (Promega) following the supplier specifications. Real-time quantitative PCR analyses were performed in a LightCycler 480 System (Roche) following the manufacturer's instructions and using pre-designed TaqMan Assays-on-Demand by Applied Biosystems.

The reference genes used for the study were Peroxisome proliferator-activated receptor γ (*Pparg*), Mm00440940_m1; CCAAT-enhancer-binding protein α (*Cebpa*), Mm00514283_s1; Adipose triglyceride lipase (*Pnpla2*),

Mm00503040_m1; Hormone-sensitive lipase (*Lipe*), Mm00495359_m1; Fatty acid synthase (*Fasn*), Mm00662319_m1 and Nuclear factor-κB (*Nfkb*), Mm00479807_m1. All mRNA levels were normalized by the housekeeping gene *Gapdh* obtained from Applied Biosystems. Samples were analyzed in triplicate. The relative expression level of each gene was calculated by the 2−ΔΔCT method [24].

Statistical analysis

All the results are expressed as mean ± standard deviation (SD) of the mean. The statistical analyses were performed using SPSS Statistics 15.0 software. Statistical significance of differences among the groups was evaluated using ANOVA test followed by the Dunnett's post hoc test. A level of probability of $p < 0.05$ was set as statistically significant.

Results

Effects of kaempferol on 3T3-L1 adipocyte viability

To address the cytotoxic effect of kaempferol on 3T3-L1 cells, an ATP-based assay was performed to measure cell viability. 3T3-L1 cells were treated with 20, 40, 60, 80, 100, 120, 150, or 170 μM kaempferol for 24, 48, and 72 h. The results indicated that kaempferol showed a time and dose-dependent effect on adipocytes viability, which seems to promote cell damage at a concentration higher than 80 μM at 72 h (Fig. 1). This effect is exacerbated at earlier times such as 24 h and 48 h, when 100, 120, 150, or 170 μM kaempferol were incubated. In contrast, 60 μM kaempferol stimulation did not affect cell viability at 24 h, 48 h nor 72 h (Fig. 1). Based on these results, we tested 60 μM kaempferol on lipid accumulation and adipogenic differentiation.

Effect of kaempferol on lipid accumulation

The effect of kaempferol on lipid accumulation in 3T3-L1 adipocytes was examined using Oil Red O. When cells achieved the ideal confluency, adipocyte differentiation was induced for 7 days with kaempferol or vehicle control (DMSO). Adipocyte differentiation was detected by cellular uptake of the oil red stain, which was induced by treatment with an adipogenic cocktail (positive control) (Fig. 2). Our results demonstrated a reduction in the number of positively stained cells and staining per cell at day 0, when compared to positive control incubated with the adipogenic cocktail containing 60 μM kaempferol (Fig. 2). This result suggests a powerful inhibitory effect on the adipogenesis process.

To quantify lipid accumulation in cells oil red was extracted and measured in an absorbance reader (Fig. 2). Kaempferol (60 μM) treatment on day 0 (when the adipogenic cocktail was first added) caused a 62% significant decrease in absorbance suggesting reduction in the total lipid content in cells ($n = 6$/group) ($p < 0.001$; Fig. 2). Also, 60 μM kaempferol exposure displays a 39% decrease in the intracellular lipid content when was added on the day 12; however, no differences were observed when compared with control cells ($n = 6$/group) ($p = 0.256$; Fig. 2).

Effects of kaempferol on adipogenesis markers expression

Adipocyte differentiation is mainly regulated by *Pparg* and *Cebpa* genes. We found that 60 μM kaempferol treatment on day 12 of adipocyte differentiation significantly decreased the mRNA expression of *Cebpa* when compared with kaempferol-non treated cells ($n = 6$/group) ($p < 0.05$; Fig. 3).

Effects of kaempferol on lipid metabolism gene expression

Moreover, the effects of kaempferol on the mRNA expression of key lipolysis regulatory enzymes were examined. As shown in Fig. 4, *Pnpla2* mRNA levels were significantly upregulated in cells treated with 60 μM kaempferol ($n = 6$/group) ($p < 0.01$). Likewise, a significant increase in the mRNA expression of *Lipe* was found in kaempferol-treated cells compared with the positive control on day 12 ($n = 6$/group) ($p < 0.01$). Finally, no statistical differences were found in *Fasn* mRNA expression levels (Fig. 4).

Discussion

Adipose tissue accumulation is a typical form of metabolic failure in obesity which includes, in part, two main metabolic and cellular processes, an increase in the amount of lipids stored inside the adipocytes and an increase number of cells (adipogenesis) [4, 25]. Potential pharmacologic strategies to decrease fat accumulation and reduce body weight have been unsuccessful, in part, by their negative side effects in metabolic relevant organs. Therefore, a natural compound capable of modulating these two molecular mechanisms might improve substantially the preventive and therapeutic approaches to treat and revert adipose tissue accumulation in obesity [4].

The current data show that 60 μM kaempferol mitigated the conversion from pre-adipocytes to mature adipocytes, suggesting an anti-adipogenic effect in 3T3-L1 cells. In addition, we identified that kaempferol is an efficient flavonoid to selectively downregulate the *Cebpa* mRNA levels with no changes in the *Pparg* expression. Previous studies have shown the notable role of PPARγ and C/EBPα as key regulating factors during adipogenesis, mainly regarding their crucial

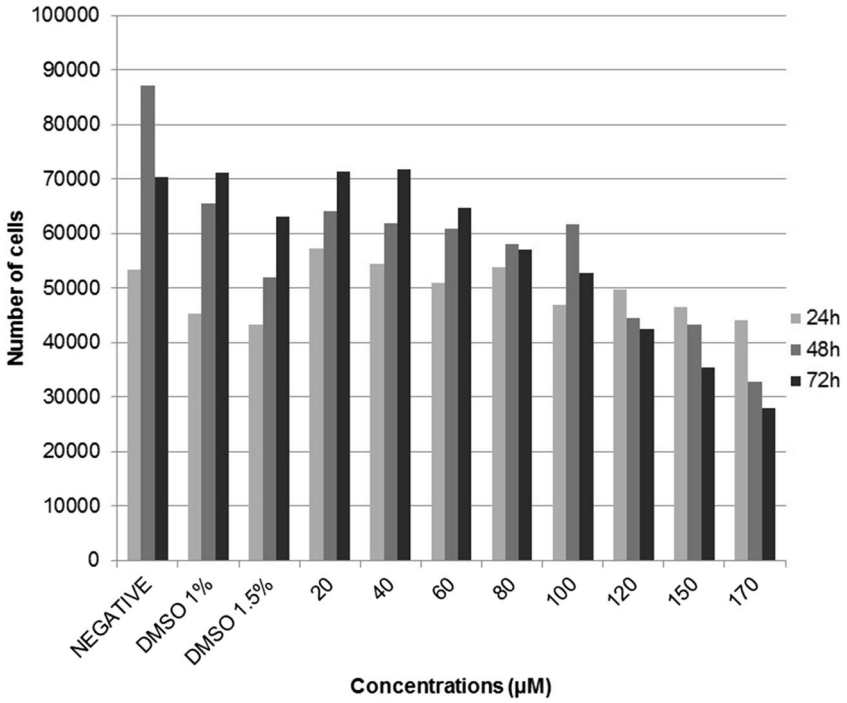

Fig. 1 Kaempferol effect on 3T3-L1 cells cytotoxicity at 24, 48, and 72 h after treatment. Cell viability was determined by the luciferin/luciferase assay

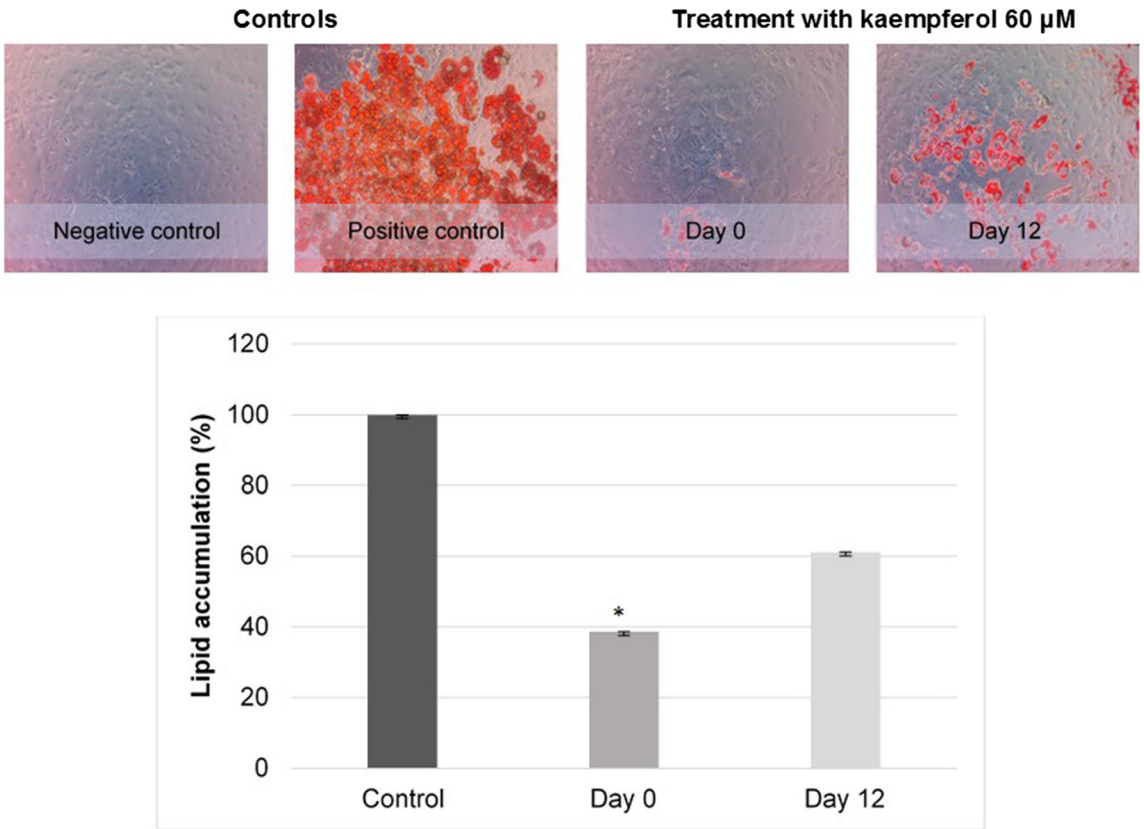

Fig 2 Kaempferol inhibited adipocyte differentiation and induction of lipolysis in 3T3-L1 cells. Results are expressed as percentage of lipid accumulation regarding positive control of differentiated cells without treatment (100%). Results represent the mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of treatment groups compared with control group ($n = 6$/group) *$p < 0.05$

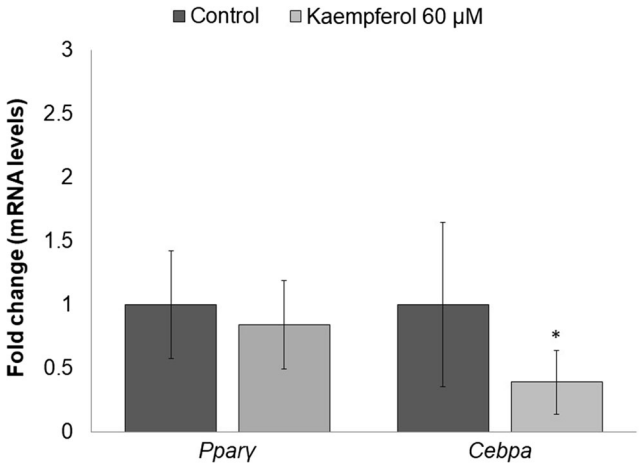

Fig 3 Kaempferol effect on mRNA expression of *Pparg* and *Cebpa* in 3T3-L1 cells. Results are expressed as fold changes compared to housekeeping gene (*GADPH*) and showed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze the differences in the mean of treatment group compared with control group (normalized to 1) ($n = 6$/group) *$p < 0.05$

role in the transcriptional activation of adipocyte differentiation gene profile [7]. In this context, a regulation in the expression of these factors can modulate the differentiation capacity of adipocytes [26]. Thus, our results add scientific evidence supporting the role of kaempferol as a pivotal molecule to modulate the adipogenic pathway by its selective effects on *Cebpa* mRNA downregulation. Our results correlate with previous findings showing inhibition of cell differentiation and *Cebpa*, *Pparg*, and *Srebp1c* mRNA downregulation following 10 or 40 μM kaempferol treatment for 7 days using the 3T3-L1 cell line model [11]. Likewise, Yeon-Joo et al. [23] reported that 3T3-L1 cells treated with 30 μM kaempferol for 8 days, reduced significantly lipid accumulation and protein

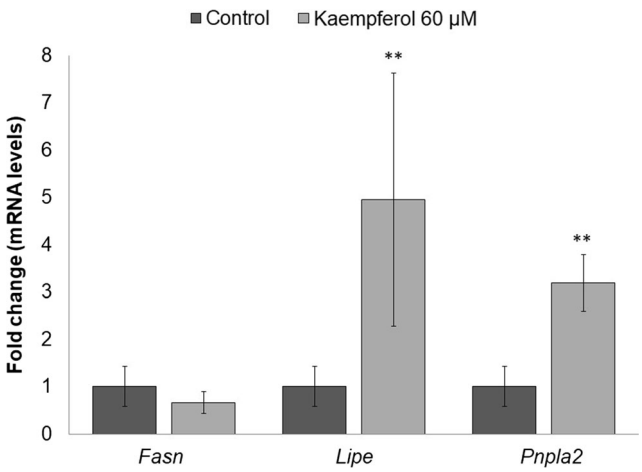

Fig 4 Kaempferol effect on mRNA expression of *Fasn*, *Lipe*, and *Pnpla2* in 3T3-L1 cells. Results are expressed as fold changes compared to housekeeping gene (*GADPH*) and shown as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze the differences in the mean of treatment group compared with control group (normalized to 1) ($n = 6$/group) **$p < 0.01$

expression levels of the PPARγ and C/EBPα adipogenic markers. Although our results and others have shown the anti-adipogenic effect of kaempferol by its inhibition of two key adipocyte differentiation genes (*Pparg* and *Cebpa*), it is tentative to speculate that additional genes, such as *Cebpb*, might be potentially involved in this process. Nevertheless, it is important to highlight that C/EBPα is a transcription factor activated by C/EBPβ and expressed throughout the differentiation process. Furthermore, there is evidence that C/EBPα could be the key responsible for the adipogenesis process [27]. Finally, it is still unknown if kaempferol concentration might be found in animal models or humans at low concentrations as we tested in our in vitro model. For instance, the mean intake of kaempferol in humans is 14.97 mg/day which allow a plasmatic concentration up to 57.86 nmol/l [28]. In vitro studies have shown a dose-dependent effect of kaempferol (from 10 to 40 μM) on decreasing lipid accumulation during adipocyte differentiation [11]. Nevertheless, other authors have not found anti-adipogenic effect of kaempferol at lower concentrations, for instance 7.5, 15, and 30 μM [23], in contrast with 60 μM kaempferol concentration of our study. In any case, in vivo studies are required to translate these results to clinical trials in order to identify a possible treatment to prevent and control obesity.

Lipolysis is one of the metabolic pathways playing an important role in the hydrolysis of triglycerides to fatty acids and glycerol, to cover the body energy requirements [29]. Of note, lipolysis activation leads to a reduction of fat content stored in the adipose tissue [29], which has become a potential pharmacologic candidate to be targeted. During lipolysis two main enzymes are involved including the adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), encoded by the *Pnpla2* and *Lipe* genes, respectively. The ATGL enzyme is responsible for the first step in the hydrolysis of triglycerides to diglycerides and free fat acids which later on, become hydrolyzed through HSL activity [12, 30]. Our data show that in addition to inhibit adipocyte differentiation, kaempferol reduces intracellular lipid content in mature adipocytes by increasing the expression of lipolysis gene profile. In specific, we added scientific evidence showing positive modulation of Pnpla2 and *Lipe* transcriptional gene expression by kaempferol. Recent studies support our findings, by showing that microarray analysis of 3T3-L1 cells treated with 40 μM kaempferol during 10 days displays an increased expression of genes involved in lipolysis, such as the lipolysis-stimulated receptor gene (*Lsr*) [20]. Our data correlates with this scenario during the 60 μM kaempferol treatment.

In summary, our research provides evidence that the treatment with kaempferol reduces efficiently the lipid accumulation in adipocytes presumably by downregulation of the expression of *Cebpa* and increasing the transcriptional gene profile involved in lipid metabolism without inhibitory effect on

the *novo* lipogenesis, suggesting a potential candidate to modulate body weight during obesity.

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